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The role of CD44H molecule in the interactions between human monocytes and pancreatic adenocarcinoma-derived microvesicles

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Abstract

Introduction. CD44H is a transmembrane molecule important for cell-cell and cell-extracellular matrix interactions. In monocytes, CD44H is implicated in phagocytosis of particles coated by hyaluronan (HA). HA fragments were shown to induce chemokine secretion by monocytes. Tumour derived microvesicles (TMVs), which are small membrane fragments derived from tumour cells can carry fragments of HA. The aim of the study was to examine whether monocyte's CD44H is involved in the engulfment of pancreatic adenocarcinoma-derived microvesicles and in the production of chemokines induced by TMVs.

Materials and methods. TMVs engulfment and chemokines' secretion stimulated with TMVs were determined in control human monocytes and cells incubated with anti-CD44H monoclonal antibody (mAb) by flow cytometry and ELISA, respectively. Phosphorylation of STAT3, transcription factor essential for chemokines' production and CD44 signal transduction, was determined by Western blotting.

Results. Blocking of CD44H by anti-CD44H mAb on monocytes decreased the engulfment of TMVs and the secretion of CCL4 and CCL5, but had no effect on CCL2, CCL3 and CXCL8. STAT-3 phosphorylation in monocytes incubated with TMVs after CD44 blocking was also reduced.

Conclusion. The results suggest that tumour-derived microvesicles (TMVs) may carry bioactive cargo(s) which induces STAT3 dependent signalling pathway in human monocytes *via* CD44 molecules. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 1, 28–34)*

Key words: CD44; human monocytes; HPC-4 cells; tumour-derived microvesicles; chemokines; STAT3 phosphorylation; flow cytometry

Introduction

CD44 is a transmembrane glycoprotein expressed on different cell types, including epithelial [1, 2], hematopoietic and cancer cells [3–6]. CD44 is expressed mainly in a short, standard form (CD44s or CD44H) or in alternatively spliced variant forms (CD44v) [7, 8]. Expression of CD44H on monocytes is very high (above 90%) [9]. CD44v is almost absent on monocytes of healthy humans; however, its expression (CD44v3, -v6, -v7) is upregulated in inflammatory diseases (*e.g.* systemic *lupus erythematosus,* inflammatory bowel disease) [10, 11], malignancies (*e.g.* CD44v3, v6, v7, v10) [9, 12–14] or co-cultures of monocytes with tumour cells (*e.g.* pancreatic adenocarcinoma cell line, CD44v6, v7/8) [15].

The increased expression of CD44v molecules on cancer cells is usually associated with upregulation of tumour growth, metastasis formation and poor prognosis in cancer patients [14]. CD44 molecule is

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important in cell-cell and cell-extracellular matrix interactions, as CD44 is a receptor for hyaluronan (hyaluronic acid, HA), collagens, osteopontin, serglycin, fibronectin and laminin [16]. HA carried by tumour cells seems to be, at least in part, responsible for the stimulation of cytokines and chemokines production by monocytes [17, 18]. Apart from direct stimulation by tumour cells (*e.g. via* HA and other ligands), monocytes may be stimulated by tumour-derived microvesicles (TMVs) [19]. TMVs are small membrane fragments released by tumour cells during cell proliferation, migration, activation and apoptosis [20]. TMVs express CD44s and CD44v and carry HA [19, 21]. TMVs may mimic activity of tumour cells, as they have been shown to induce cytokines' (TNF, IL-10, IL-12), chemokines' (CXCL8, CCL2, CCL3, CCL4 and CCL5) and reactive oxygen intermediates (ROI) production by monocytes [19, 22]. It was reported that TNF production in monocytes was CD44-dependent [19] and that IL-10 production by classical monocytes was induced by low molecular weight hyaluronan carried by TMVs [21]. HA-CD44 interaction promotes phosphorylation of STAT3 [23]. CD44 is also described as fully competent phagocytic receptor able to trigger engulfment of large particles by macrophages [24].

The current study was designed to extend the knowledge on the role of CD44H in monocyte-TMVs interactions. We focused on the engulfment of TMV derived from HPC-4 cell line (TMV_{HPC}) and the secretion of selected chemokines (CXCL8 (IL-8), CCL-2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES)) previously described to be induced by TMVs [22]. The present study shows that blocking of monocytic CD44H molecule with anti-CD44 monoclonal antibody (mAb) reduced TMV_{HPC} engulfment and decreased secretion of CCL4 and CCL5 but not CCL2, CCL3 and CXCL8. We also proved the contribution of STAT3 in signalling pathway initiated by CD44H-TMVs interaction.

Materials and methods

Isolation of tumour derived microvesicles (TMVs). TMVs were obtained from the HPC-4 cell line (human pancreatic adenocarcinoma) TMV_{HPC}, [25] as described previously [26]. Briefly, cells were cultured by bi-weekly passages in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 5% FBS (foetal bovine serum, PAA Laboratories, Pasching, Germany). The cell line was regularly tested for *Mycoplasma sp.* contamination by using the PCR-ELISA kit according to the manufacturer's protocol (Roche, Mannheim, Germany). For the final cultures, FBS centrifuged at 50000 *g* was used (for 1 h at 4°C). Supernatants from well-grown cell cultures were collected and spun down at 2000 *g* for 20 min at room temperature (RT) to remove cellular debris. Then, supernatants were again centrifuged at 50000 *g* for 1 h at 4°C. Pellets were washed several times to remove FBS and were finally resuspended in serum-free RPMI 1640 medium. Quantification of TMV_{HPC} proteins was evaluated by the Bradford method (BioRad, Hercules, CA, USA). TMV_{HPC} were tested for endotoxin contamination by the Limmulus test according to the manufacturer's instruction (Charles River Laboratories, Inc., Wilmington, MA, USA) and stored at –20°C.

Isolation and culture of monocytes. Human peripheral blood mononuclear cells were isolated from EDTA-blood of healthy human donors by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from mononuclear cells by counter-flow centrifugal elutriation with a JE-5.0 elutriation system equipped with a 5 ml Sanderson separation chamber (Beckman, Palo Alto, CA, USA), as previously described [27]. Monocytes were suspended in RPMI 1640 culture medium supplemented with L-glutamine (Sigma) with gentamycin (25 μg/ml). Purity of isolated monocytes was above 95% as judged by staining with anti-CD14 mAb (BD Biosciences Pharmingen, San Diego, CA, USA) and flow cytometry analysis (FACS Calibur, BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Monocytes (1×10^6/ml) were cultured with TMV_{HPC} (30 μg/ml) in low attachment culture plates (Corning Inc., Corning, NY, USA) in RPMI 1640 medium supplemented with 10% FBS (centrifuged as above) as described before [19].

Engulfment of TMVs by monocytes. TMV_{HPC} were incubated for 5 min with red PKH26 dye (Sigma) according to the manufacturer's instructions. Next, TMV_{HPC} were washed with 1% bovine serum albumin (BSA) and several times with serum-free RPMI 1640 medium. Monocytes (1×10^6/ml) were incubated with anti-CD44 mAb (10 μg/ml, clone SFF-2, BenderMedsystem, Vienna, Austria) or appropriate IgG1 isotype control (10 μg/ml Bender Medsystem) for 2 h at 37°C followed by washing and incubation with a fluorescent dye PKH26-labelled TMV_{HPC} (30 μg/ml) (30 min to 24 h at 37°C in serum-free medium). Binding of PKH26-labelled TMV_{HPC} to control and CD44-blocked monocytes was determined by flow cytometry analysis of red fluorescence intensity (emission at 567 nm) and calculation of the percentage of positive cells. Vital dye crystal violet was used for quenching extracellular fluorescence [28].

Determination of chemokines' secretion by monocytes incubated with TMVs after blocking of CD44 molecule on monocytes. To determine its role in monocyte-TMV_{HPC} interactions, the blocking mAb against CD44H was used. Monocytes were incubated on 96 microwell plates with

anti-CD14 mAb (10 μg/ml, clone MY4, Coulter Corp., Miami, FL, USA) or appropriate IgG1 isotype control for 2 h at 37°C. Then, monocytes were washed and cultured with TMV_{HPC} (30 μg/ml) for 18 h. Next, the supernatants were collected and chemokines' (CXCL8, CCL2, CCL3, CCL4 and CCL5) concentration was assessed by the FlexSet method (BD Biosciences Pharmingen) according to the manufacturer's protocol. The FlexSet beads were discriminated in FL-4 and FL-5 channels, while the concentration of specified chemokine was determined by the intensity of FL-2 fluorescence. The concentration of chemokines was computed by using the respective standard reference curve and FCAP Array software (BD Biosciences). For all the tested chemokines the detection level was 10 pg/ml.

Western blotting. Monocytes were preincubated in the medium alone or with anti-CD44 mAb (10 μg/ml) for 2 h followed by washing and incubation with TMV_{HPC} (30 μg/ml) for 30 min (37°C, 5% CO_2), then lysed in M-Per lysing buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (Sigma). The extracted proteins (20 μg) were loaded on 4% loading gel, electrophoresed in 12% SDS gel, and transferred to the polyvinylidene fluoride membranes (Immune-blot PVDF, 2 μm, BioRad). Phosphorylation of STAT-3 (Signal Transducers and Activators of Transcription, Tyr 705, #9131) protein was detected with rabbit polyclonal anti-phospho-STAT-3 antibody (Cell Signaling Technology, Beverly, MA, USA) and with horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). The equivalence of protein loading was evaluated by treating the membranes with stripping buffer (Restore Western Blot Stripping Buffer, Pierce) and incubation with total anti-STAT-3 antibody (clone #9132, Cell Signaling). Blots were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce), dried and subsequently exposed to HyperFilm (Amersham Life Science, Little Chalfont, UK).

Statistical analysis. Statistical analysis was performed by nonparametric Mann-Whitney test. Differences were considered significant at $p < 0.05$.

Results

The role of CD44H in TMVs engulfment by monocytes

Engulfment of TMV_{HPC} was significantly reduced when incubation with TMV_{HPC} was preceded by blocking CD44 on monocytes for 2 h. We did not observe significant changes after 30 min (data not shown); however, after 2 h and 24 h, the TMV_{HPC} engulfment was reduced by half (Fig. 1). Appropriate isotype control did not diminish TMV_{HPC} engulfment (data not shown).

CD44H is important for chemokines' secretion by monocytes

The release of CCL5 (Fig. 2A) and CCL4 (Fig. 2B) from human monocytes incubated with TMV_{HPC} overnight (18 h) was decreased when CD44H, but not CD14 (data not shown), was blocked by the preincubation of cells with appropriate mAb. We did not observe significant changes in the levels of CCL2 (Fig. 2C), CCL3 (Fig. 2D) and CXCL8 (Fig. 2E) after CD44 blocking.

The role of CD44H molecule in signal transduction

TMV_{HPC} induced phosphorylation of STAT-3 proteins in monocytes. Phosphorylation of STAT-3, as determined by Western blotting, was reduced when monocytic CD44H was blocked with anti CD44mAb before stimulation with TMV_{HPC}. Results of one representative experiment out of three performed is presented in Figure 3.

Discussion

Monocytes and TMVs derived from CD44-positive tumour cell lines express CD44H molecules [29, 30]. TMVs can also carry HA derived from cancer cells [21]. Interaction between monocytes' CD44 molecule and HA carried by TMVs results in the activation of monocytes [19, 21]. Previously, we showed that monocytes activated with TMVs secreted more TNF, IL-10 and IL-12p40 and expressed higher levels of these cytokines' mRNA compared to the control [19]. The inhibitory effect of anti-CD44H mAb provided evidence that this molecule is important for TNF secretion by monocytes stimulated with TMVs [19]. Current data add to the knowledge about the role of CD44 in interactions between monocytes and TMVs. CD44 molecule seems to be important in the process of TMV_{HPC} engulfment, which is strongly inhibited by blocking monocytic CD44 with anti-CD44 mAb. This observation is in concordance with the report that demonstrated inhibition by anti-CD44 mAb of erythrocytes' phagocytosis by murine macrophages [31]. Moreover, CD44 is considered as a phagocytic receptor that effectively recognizes and ingests HA-coated particles [24]. CD44 not only mediates the phagocytic mode of internalization but it also facilitates the HA-controlled uptake of a gene vector in CD44 positive tumour cancer cell lines *via* micropinocytosis [32]. The mechanism by which CD44 is engaged in this process is unclear; however, its involvement in the first step of interaction (binding) was suggested [33]. In our current study, the engulfment of TMV_{HPC} was not completely reduced by anti-CD44 mAb, which may suggest other mechanisms and sur-

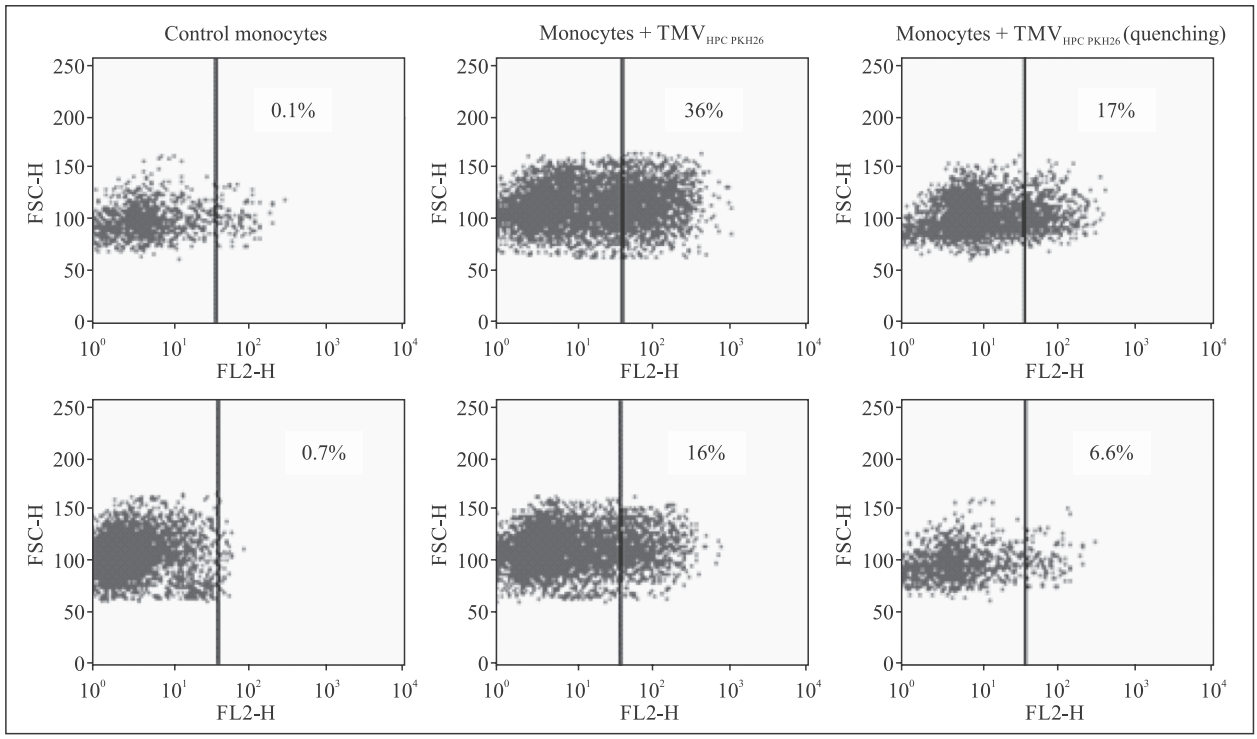

Figure 1. Transfer of PKH26 labelled TMV_{HPC} to monocytes in the absence (upper dot plot panel) and presence (lower dot plot panel) of anti CD44H mAb (10 μg/ml). Monocytes were exposed to TMV_{HPC} for 24 h and incubated either in the medium alone or with crystal violet (right panel). One representative experiment of four independent experiments is presented. Flow cytometry was performed as described in Methods.

face molecules to be involved in their internalization, *e.g.* phosphatidylserine [34]. To our best knowledge, the presented data, for the first time, provide the evidence of the role of CD44 in the engulfment of TMVs in a manner similar to that suggested for particles coated with HA [32].

McKee *et al.* [17] showed that low molecular weight HA induced the expression of mRNA for CCL2, CCL3, CCL4, CCL5 and CXCL8 in alveolar macrophages [17] and that small fragments of HA were able to induce secretion of CCL2, CCL3, CCL4 and CCL5 by these cells. These authors also demonstrated that anti-CD44 mAb completely blocked HA binding and significantly inhibited HA-induced expression of CCL4 and CCL5 in this cell type [17]. In parallel, we reported that TMVs of different origin (lung, pancreas and colon cancer cell lines) induced expression of chemokines mRNA followed by secretion of chemokines by human monocytes [22]. Keeping in mind that TMV_{HPC} carry low molecular weight HA [21], we blocked CD44H on human monocytes and observed the reduction of CCL4 and CCL5 secretion by monocytes. The incomplete reduction in chemokines secretion that we have seen may be a result of the contribution of other HA receptors, which were not blocked by the anti-CD44H mAb used. Levesque *et al.* described that blood monocytes up-regulated CD44v6 and v9 expression after *in vitro* culture [35]. Also, co-culture with tumour cells induced expression of CD44v6 and v7/8 on human monocytes [15]. Thus, in our study, the mAb specific for CD44H blocking may have not blocked CD44v, which in turn could have resulted in HA binding. Also, other molecules, such as TLR4, have to be taken under consideration as HA receptors [36]. Moreover, lipids, heat shock proteins [37] or nucleotides [38] carried by TMVs may be involved in the induction of chemokines. Other components of TMVs may address the question about the role of CD44H in the signalling pathway for the chemokines which secretion was unaffected by anti-CD44 mAb and TMVs (CXCL8, CCL2 and CCL3). Another possible explanation is horizontal transfer of chemokines by TMVs, as TMVs are a storage pool for CC and CXC chemokine families [22, 39].

It was reported that phosphorylation of STAT3 transcription factor is important for the synthesis of chemokines by monocytic cells [40–42] or vascular smooth muscle cells [43]. TMV_{HPC}-induced STAT-3 phosphorylation in monocytes was shown to be involved in TNF and IL-10 gene transcription [19].

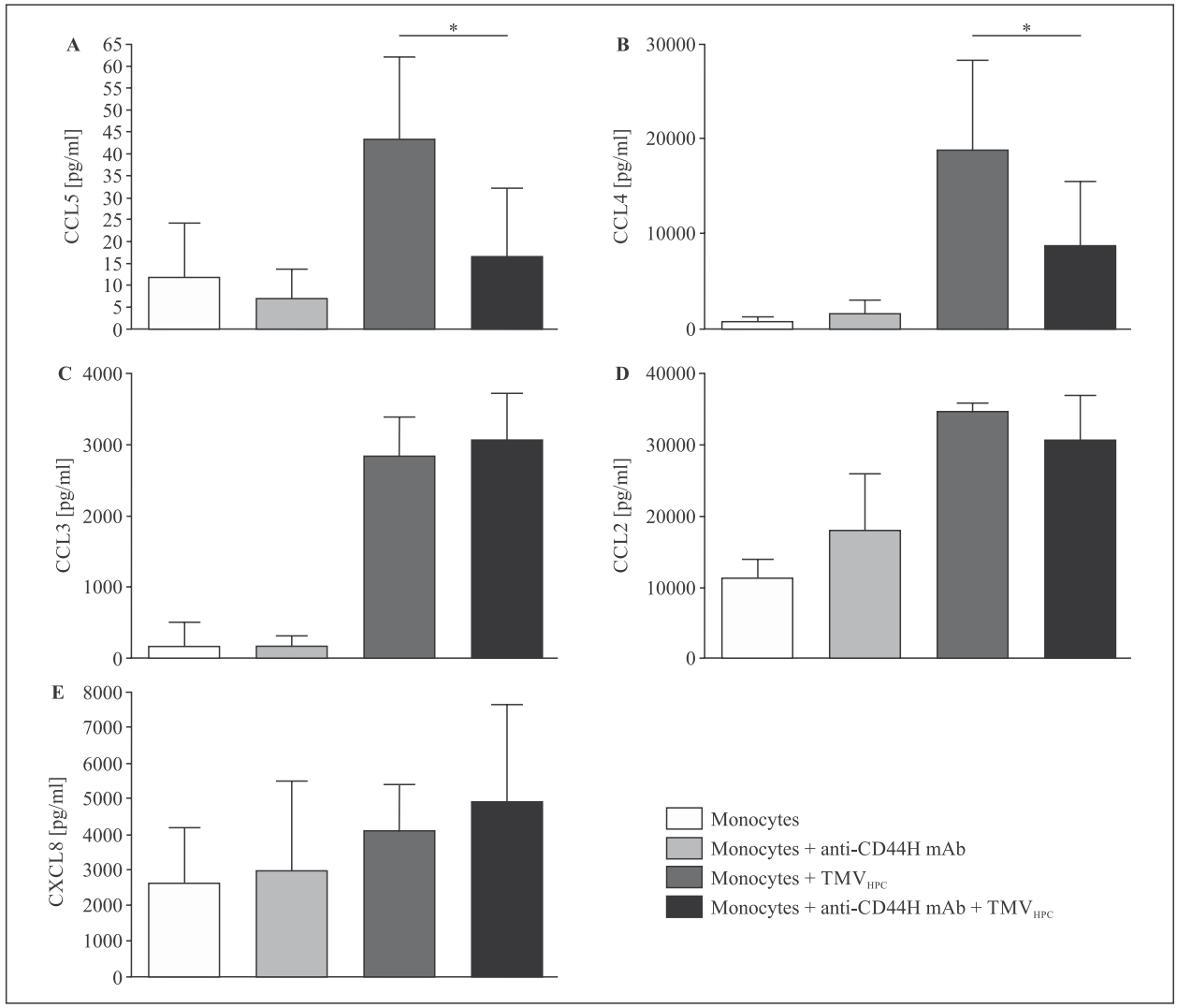

Figure 2. Secretion of chemokines by monocytes stimulated with TMV_{HPC} alone or in the presence of anti-CD44H mAb (10 μg/ml). A-CCL5, B-CCL4, C-CCL3, D-CCL2, E-CXCL8. Concentration of chemokines was measured by the FlexSet method as described in Methods. Data presented as mean ± SD of five independent experiments. * $p < 0.05$.

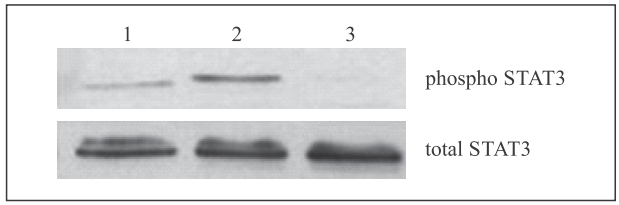

Figure 3. Expression of phosphorylated (Tyr 705) and total STAT-3 determined by Western blotting in unstimulated monocytes (lane 1) or in monocytes stimulated for 30 min with TMV_{HPC} (lane 2) alone or after preincubation with anti-CD44H mAb (lane 3).

Reduction in STAT3 phosphorylation after CD44H blocking may suggest that this signalling pathway is induced in monocytes by TMV_{HPC}. Taken together, the results of this study point out to the role of CD44 in TMV_{HPC} –monocyte interaction. TMV_{HPC} carry information which is, at least partially, passed through the signalling pathway initiated by CD44 molecules. TMV_{HPC}, may be considered as an important chemokine secretion trigger during tumour progression. The presented data imply that TMVs may play a role in the communication between various types of cells, including tumour cells, at local and distant levels.

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Competing interests

The authors have no conflicts of interest.

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